university of groningen

University Medical Center Groningen

University of Groningen

The role of physiology in the divergence of two incipient cichlid species

Dijkstra, P. D.; Wiegertjes, G. F.; Forlenza, M.; van der Sluijs, I.; Hofmann, H. A.; Metcalfe, N. B.; Groothuis, T. G. G.

Published in:
Journal of Evolutionary Biology

DOI:
10.1111/j.1420-9101.2011.02389.x

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2011

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Dijkstra, P. D., Wiegertjes, G. F., Forlenza, M., van der Sluijs, I., Hofmann, H. A., Metcalfe, N. B., & Groothuis, T. G. G. (2011). The role of physiology in the divergence of two incipient cichlid species. *Journal of Evolutionary Biology*, *24*(12), 2639-2652. https://doi.org/10.1111/j.1420-9101.2011.02389.x

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 06-05-2023

JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2011.02389.x

The role of physiology in the divergence of two incipient cichlid species

P. D. DIJKSTRA*†‡, G. F. WIEGERTJES§, M. FORLENZA§, I. VAN DER SLUIJS¶, H. A. HOFMANN‡**, N. B. METCALFE† & T. G. G. GROOTHUIS*

*Behavioural Biology Research Group, University of Groningen, Haren, The Netherlands
†Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow, UK
‡Section of Integrative Biology, The University of Texas at Austin, Austin, TX, USA
§Department of Animal Sciences, Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, Wageningen, The Netherlands
¶Department of Biology, McGill University, Montreal, PQ, Canada
**Institute for Cellular and Molecular Biology, Institute for Neuroscience, The University of Texas at Austin, Austin, TX, USA

Keywords:

11-Ketotestosterone;
carotenoids;
cichlid;
immune function;
male–male competition;
oxidative stress;
steroid;
teleost;
testosterone.

Abstract

Sexual selection on male coloration has been implicated in the evolution of colourful species flocks of East African cichlid fish. During adaptive radiations, animals diverge in multiple phenotypic traits, but the role of physiology has received limited attention. Here, we report how divergence in physiology may contribute to the stable coexistence of two hybridizing incipient species of cichlid fish from Lake Victoria. Males of *Pundamilia nyererei* (males are red) tend to defeat those of *Pundamilia pundamilia* (males are blue), yet the two sibling species coexist in nature. It has been suggested that red males bear a physiological cost that might offset their dominance advantage. We tested the hypothesis that the two species differ in oxidative stress levels and immune function and that this difference is correlated with differences in circulating steroid levels. We manipulated the social context and found red males experienced significantly higher oxidative stress levels than blue males, but only in a territorial context when colour and aggression are maximally expressed. Red males exhibited greater aggression levels and lower humoral immune response than blue males, but no detectable difference in steroid levels. Red males appear to trade off increased aggressiveness with physiological costs, contributing to the coexistence of the two species. Correlated divergence in colour, behaviour and physiology might be widespread in the dramatically diverse cichlid radiations in East African lakes and may play a crucial role in the remarkably rapid speciation of these fish.

Introduction

Across vertebrates, there are numerous mechanisms that can influence the evolution of colour diversity. Both temporal and spatial niche partitioning (Schluter, 2000; but see Vandermeer *et al.*, 2002) and balancing and frequency-dependent selection (Sinervo & Calsbeek, 2006; Gray & McKinnon 2007) have been invoked as mechanisms that can promote coexistence of colourful conspecific morphs or sympatric sister species. Sexual selection on male secondary sexual traits can also be a powerful force driving population differentiation and maintaining reproductive isolation between sympatric species (West-Eberhard, 1979). The exceptionally diverse haplochromine cichlid fishes in the East African Great Lakes provide textbook examples of rapid speciation by sexual selection (Kocher, 2004; Seehausen, 2006; Salzburger, 2009). The species flocks in these lakes are characterized by closely related sympatric species or conspecific colour morphs that are typically ecologically similar but differ greatly in male nuptial colouration. The coexistence of ecologically similar haplochromine species, and the maintenance of phenotypic bimodality

Correspondence: Dr. Peter D. Dijkstra, The University of Texas at Austin, Section of Integrative Biology, 1 University Station–C0930, Austin, TX 78712, USA.
Tel.: +1 512 475 7318; fax: +1 512 475 6754;
e-mail: pddijkstra@gmail.com

at locations where they hybridize, has intrigued many researchers and provides an ideal scenario to investigate potential mechanisms for the persistence of phenotypic variation (for reviews, see Salzburger & Meyer, 2004; Genner & Turner, 2005; Seehausen, 2009).

In haplochromine cichlids, several forces of selection have been identified as potential drivers of speciation, including mate choice, intrasexual competition and ecological resource competition (for reviews, see Kocher, 2004; Genner & Turner, 2005; Seehausen, 2009). Haplochromine social systems exhibit intense male territoriality and female-only parental care, factors that are generally thought to be conducive of strong sexual selection. Because colour is an important communication cue in competition for territories, which are required to gain access to females (Maan *et al.*, 2004; Pauers *et al.*, 2008), male–male competition can exert selection on male colouration in haplochromines (Seehausen & Schluter, 2004). Interestingly, sibling species or conspecific colour morphs often differ in the intensity of male territoriality (e.g. Dijkstra *et al.*, 2009a). However, despite the common variation in male aggression in these species, the potential role of physiological differences facilitating phenotypic variation has rarely been addressed in this group.

It seems likely that physiology is often intimately linked to colour evolution. Across vertebrate species, key physiological attributes such as oxidative stress physiology and immunity are often correlated with colour via pleiotropic mechanisms and/or pigment allocation strategies (McGraw, 2005; Griffith *et al.*, 2006; Ducrest *et al.* 2008). Oxidative stress physiology is a central mediator in the evolution of life-history trade-offs, such as immunity and sexual ornamentation (Dowling & Simmons, 2009; Monaghan *et al.*, 2009). Following the suggestion of Von Schantz *et al.* (1999) that sexually selected traits might advertise oxidative stress levels and that this might be linked to genetic variation in fitness, there has been a great interest in how oxidative stress may influence expression of sexually selected traits (Dowling & Simmons, 2009; Monaghan *et al.*, 2009). Another important physiological trait is the ability to mount an immune response against pathogens and parasites (Folstad & Karter, 1992; Kurtz *et al.*, 2007).

Whereas physiological performance can be under disruptive selection due to local environmental adaptation, physiological processes in turn can exert or influence selection on other fitness-related traits and vice versa (e.g. androgens and immune function mutually affect one another). The physiological basis of life-history trade-offs is well recognized in evolutionary biology (e.g. Mills *et al.*, 2008; reviews: Zera & Harshman, 2001; Zera *et al.*, 2007; McKinnon & Pierotti, 2010). However, selection on physiological traits is rarely considered in the haplochromine cichlid species flock. In addition, physiological performance can be an important factor in balancing and frequency-dependent selection. For example, red-headed male morphs of the Gouldian finch (*Erythrura gouldiae*) aggressively dominate black-headed morphs in competition for essential resources, but the former display higher levels of testosterone and corticosterone along with reduced cell-mediated immune responses as competition increased. Thus, disruptive selection on physiology may be a significant factor in evolutionary diversification both in terms divergent selection on suites of correlated traits, and as a component in balancing and frequency-dependent selection (Zera *et al.*, 2007; McGlothlin & Ketterson, 2008; Nosil & Harmon, 2009).

Several lines of evidence suggest that closely related cichlid species may differ in terms of physiological performance. Firstly, between species or morph variation in aggressiveness has been associated with higher androgen levels in the more aggressive species (e.g. Owen–Ashley & Butler, 2004; Korzan *et al.*, 2008). Higher androgens can in turn increase oxidative stress (Monaghan *et al.*, 2009) and suppress immune function (Kurtz *et al.*, 2007). Secondly, species may differ in circulating levels of glucocorticoids, which in addition to their role in stress physiology are intimately linked to behaviour and various other physiological functions, including immunity (for reviews, see Wendelaar Bonga, 1997; Soma *et al.*, 2008). Thirdly, the pigments (carotenoids in particular) used to produce differing colour patterns may subserve additional functions, such as neutralizing reactive oxygen species (ROS) and enhancement of the immune system (McGraw, 2005; Griffith *et al.*, 2006). Thus, variation in pigment allocation strategies across sibling species may well result in different levels of oxidative stress or immune status. Yet, these factors have rarely been considered in attempts to understand the rapid evolution of haplochromine cichlid radiations.

The haplochromine cichlid species *Pundamilia pundamilia* (Seehausen *et al.*, 1998), *Pundamilia nyererei* (Witte–Maas & Witte, 1985) and their hybrids occur at various stages of ecological and genetic differentiation around different islands along a gradient of water clarity in Lake Victoria, East Africa (for review see Seehausen, 2009). Males of *P. nyererei* are crimson dorsally (including the dorsal fin) and yellow on their flanks (referred to as 'red'). Males of *P. pundamilia* are metallic blue on the body and in the dorsal fin (referred to as 'blue'). Colour phenotype is largely genetically determined in this system (Magalhaes *et al.*, 2009). Along this gradient of water clarity, the two species go from being one species with phenotypic variation (unimodal, predominantly intermediate between red and blue) to two incipient species, and finally two reproductively isolated sister species, in what has been called a 'speciation transect' (Seehausen *et al.*, 2008). In the present study, we selected from this speciation transect two frequently hybridizing incipient species at Kissenda Island. The *Pundamilia* species at this location showed the weakest yet still significant bimodality in the distribution of male nuptial colouration and the lowest significant differentiation

at neutral loci, making them the most suitable population to investigate traits under disruptive and divergent selection (Magalhaes *et al.*, 2009). At this location, female mating preference for own colour is weakly significant, but this preference for own colour can be overridden by territory quality (Dijkstra *et al.*, 2008). Red is more aggressive (Dijkstra *et al.*, 2009a) and is more likely to defeat blue in dyadic combats (Dijkstra *et al.*, 2005). However, red does not displace blue despite its dominance advantage at this location, and throughout the lake, the geographical distribution of red fish in *Pundamilia* is nested within that of the blue colour (Seehausen & Van Alphen, 1999), indicating that the behavioural advantages enjoyed by red males may be offset by other costs. We examined whether the two species differed in aggressiveness, oxidative stress, immunity and circulating steroid levels.

There are several *a priori* reasons to hypothesize that red males experience higher oxidative stress and show a lower immune response than blue males. First, due to their relatively high level of aggression, red males likely exhibit a higher metabolic rate than blue males, possibly resulting in more oxidative stress due to the link between metabolic expenditure and production of ROS (Metcalfe & Alonso-Alvarez, 2010). Second, red males may have higher steroid levels, which may increase oxidative stress and suppress immune function (see above). Finally, we have previously shown that expressing red colouration imposes an immunological cost in red *Pundamilia* males (Dijkstra *et al.*, 2007). The nuptial dress of red males contains a far higher concentration of carotenoid pigments than that of blue males (Maan *et al.*, 2008). Because carotenoids also have antioxidant and immunostimulant properties and are thought to occur in limited supply, there is a potential trade-off between allocating carotenoids towards colouration or towards health maintenance (Lozano, 1994; Hill, 1999; Blount, 2004). Based on these reasons, we predicted that red males experience more oxidative stress than blue males, but only when colouration and aggression are intensified during territorial bouts (Dijkstra *et al.*, 2007). In a second experiment, we tested for differences in immune function by measuring the humoral immune response in fish, using a sheep red blood cell (SRBC) challenge, and blood leucocyte analyses. We predicted a lower humoral immune response in red males than in blue males. Together, our results show that red males exhibit a trade-off in terms of social status and physiology and provide a scenario through which physiology may counterbalance the advantages of increased aggressiveness.

Material and methods

Study species and housing

We studied lab-bred offspring of wild-caught *Pundamilia* fish from Kissenda Island, Lake Victoria, Tanzania, collected in 2002. Phenotypically distinct red and blue phenotypes are common around this island, with intermediate forms occurring at lower frequencies. We only used distinct red and blue phenotypes (Fig. 1, see also colour scale Dijkstra *et al.*, 2007: 4 and 5, and 0 and 1, for respectively red and blue phenotypes). For details of the lab-bred stock and general aquarium conditions, we refer to Dijkstra *et al.* (2009a). In the experiments (detailed below), males were housed individually in 25-L compartments as detailed elsewhere (Dijkstra *et al.*, 2007).

Experiment 1. Oxidative stress, aggressiveness and hormone profiles

Experimental design

We used a total of 52 adult fish (blue fish: body mass, 22.5 ± 0.7 g mean ± SE, standard length, 90.4 ± 1.0 mm, $n = 27$; red fish: body mass, 21.9 ± 0.6 g, standard length, 89.6 ± 0.8 mm, $n = 25$) in three treatment conditions. Haplochromine males occur in two flexible 'social phenotypes'; territorial, reproductively active males that are brightly coloured, and nonterritorial males, nonreproductive males that do not fully express nuptial colouration (e.g. Hofmann, 2003). These different social states are associated with different life-history traits in terms of, for example, growth rate (Hofmann *et al.*, 1999) and immunity (Dijkstra *et al.*, 2007). We manipulated social status by housing males either in social *isolation* (resulting in nonterritorial status), or in a social condition where males are 'forced' into a territorial state (Dijkstra *et al.*, 2007). We thus used two social conditions

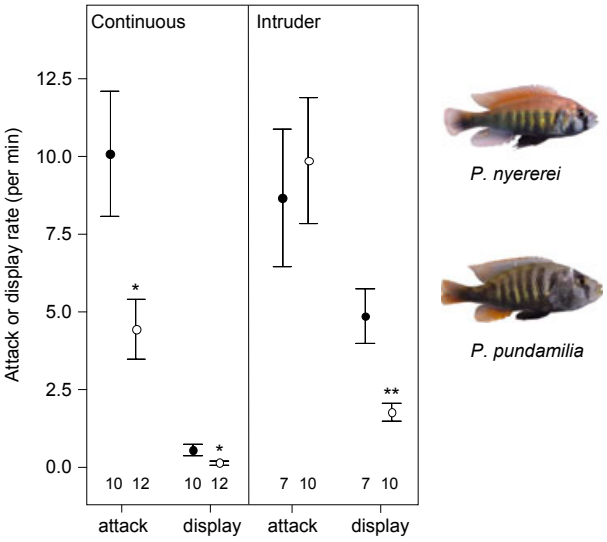

Fig. 1 Red (●) and blue males (○) differ in aggressiveness. Shown are the rates per minute for attack and display behaviour (mean ± SE) in the continuous social treatment and the intruder treatment. The significance of the species difference is indicated on top. $*P < 0.05$, $**P < 0.01$.

mimicking two types of territorial defence: ongoing territory defence against a familiar rival male (*continuous social treatment*, see Dijkstra *et al.*, 2007) and acute territory defence against an unfamiliar intruder (*intrusion treatment*, Hirschenhauser *et al.*, 2004). In all social situations, rival males were matched on standard length (difference was <10% the standard length of the smaller fish). In the *continuous social treatment*, the focal male was housed next to a rival male of the same colour – separated by a transparent and perforated partition to allow for visual and olfactory communication – to stimulate territoriality and development of nuptial colouration. Males were not exposed to males of the other colour because previous studies had shown that males of both species preferentially attack their own colour (Dijkstra *et al.*, 2009a), and our aim was to maximize territorial condition. After keeping focal males in this arrangement, we filmed focal males for a five-minute period using a Sony Handycam (DCR-SR52) and later scored their aggressive behaviour from the video recordings. A five-minute interval is sufficient to obtain highly repeatable estimates of rate of aggression (see e.g. Dijkstra *et al.* 2006). Immediately after this, we collected blood from the caudal vein using a needle and a syringe. In the *intrusion treatment*, the focal male was also housed next to a rival male of the same colour for at least a week, but this rival male was then removed, and an unfamiliar stimulus male enclosed in a transparent cylinder was introduced into the compartment of the focal male. There was a continuous flow of water over the top edge of the cylinder into the compartment of the focal male to provide olfactory stimulation. We filmed the fish for 15 min immediately from the moment the stimulus was introduced and later recorded aggressiveness. A 15-min interval was selected instead of 5 min since a pilot study indicated that behaviour directed to a novel intruder is more variable across time (unpublished data). Forty five minutes after stimulus onset, we took a blood sample from the focal male. Finally, in the *isolation treatment*, fish were housed in physical isolation and without visual or chemical contact to neighbouring fish for 1 week before blood was collected. Because isolated fish do not exhibit any social behaviour, we did not quantify their behaviour.

Immediately after the behavioural observation, we collected blood from 10 red and 12 blue males in the continuous social treatment, seven red and 10 blue fish in the intrusion treatment and eight red and five blue males in the isolation treatment. Blood was collected (20- to 100-μL) within 2 min after capturing the fish from the caudal vein using a 1-mL syringe and 25-gauge needle (length 25 mm, cat. nr. 613-0557, Neolus). Upon collection, each blood sample received a drop of heparin (<1 μL) and was immediately placed on ice until centrifuged at 9447.1 *g*, radius 5 cm, 13 000 rpm for 10 min. The plasma was then removed. To avoid repeated freeze–thaw cycles, we prepared 5–20 μL plasma aliquots and stored it at −80 °C for analysis in the oxidative stress and hormone assays.

Quantification of behaviour

An observer quantified from the video recordings the rate at which the focal male performed both display and attack behaviours towards the stimulus male. A *display* event was defined as a lateral or frontal display (Baerends & Baerends–Van Roon, 1950). During frontal displays, the focal male extends his dorsal fins, and sometimes pectoral fin and operculum as well, while facing the lateral or frontal side of the stimulus male. During a lateral display, the male extends his dorsal, anal and pelvic fins and positions himself such that his flank is in front of the head of the stimulus male. An *attack* event was defined as an individual butt or bite against the transparent partition or cylinder.

Hormone assays

We measured in duplicate circulating levels of cortisol (CORT) and two androgens, testosterone (T) and the teleost-specific 11-ketotestosterone (11-KT), in blood plasma using enzyme immunoassays (T and CORT: Assay Design, Ann Arbor, MI, USA; 11-KT Cayman Chemical Ann Arbor, MI, USA) following protocols established by Kidd *et al.* (2010). These assay systems measure both the free and bound (to steroid binding proteins) fraction. Plasma (7.2 μL) was diluted 1 : 30 with assay buffer, and the manufacturer's instructions were followed (see Kidd *et al.*, 2010). A similar protocol was used for CORT and 11-KT. The quantity of plasma used in the CORT assay was also 7.2 μL and in the 11-KT assay 3.6 μL. To assess the inter- and intra-assay variation for each EIA, the coefficient of variation was calculated (Kidd *et al.*, 2010). The intra-assay CV was 2.21%, 2.57% and 1.58% for the CORT, 11-KT and T assays, respectively. For the inter-assay variation, we assayed 13 (for CORT) and nine (for T) samples twice on separate plates. For 11-KT, we used a pooled sample of the African cichlid *Astatotilapia burtoni* on three separate plates. The inter-assay CV was 10.18%, 4.46% and 6.56% for CORT, 11-KT and T, respectively.

Oxidative stress

Normal metabolic activity is a primary source of ROS, highly reactive molecules capable of causing oxidative damage to biomolecules if not counteracted by the antioxidant system (Matsuo & Kakato, 2000). In this study, we did not measure oxidative damage directly, but measured both reactive oxygen metabolite (ROM) concentrations and overall antioxidant capacity. The balance between oxidants and antioxidants defines the level of oxidative stress of an organism (Finkel & Holbrook 2000). Therefore, we calculated the ratio of ROMs to antioxidant capacity to assess the level of oxidative stress, with higher values corresponding to higher level of oxidative damage (Costantini *et al.*, 2006; Bagni *et al.*, 2007).

Measurement of reactive oxygen metabolites

The serum concentration of ROMs (primarily hydroperoxides, ROOH) was measured by the d-ROM test (Diacron, Grosseto, Italy), as in earlier studies of oxidative stress levels in mammals (e.g. Tanganelli *et al.*, 2000), birds (e.g. Costantini *et al.*, 2006) and fish (e.g. Bagni *et al.*, 2007). For more information, see Costantini *et al.* (2006). Each serum sample (7.5 μL) was incubated in duplicate for 75 min at 37 °C with 200 μL of 0.01 M acetic acid/sodium acetate buffer, pH 4.81, v/v, containing N,N-diethyl-phenylenediamine as chromogen. Absorbance was read at 490 nm by a Biolinx plate reader. To perform the system calibration, 4.5 mM H_2O_2 as reference standard and a reagent blank were used. The intra-assay CV was 7.55%. To evaluate the inter-assay variation, 12 samples were run once on two different plates, and the inter-assay CV was 9.14%.

Measurement of antioxidant capacity

Serum antioxidant defence was measured in the form of free radical scavenging capacity, quantified as the capability to neutralize the oxidant action of HOCl using the OXY assay (Diacron, Grosseto, Italy). The serum (2 μL) was diluted 1 : 100 with distilled water. Five microliter of each diluted serum sample was incubated in duplicate for 10 min at 37 °C with 200 μL of a titrated HOCl solution as oxidant. Then, 5 μL of the same chromogenous solution used for the ROMs determination was added. Absorbance was read at 490 nm as the end-point. Calibration was achieved using a reference serum able to neutralize 440 μM HOCl. Measurements were expressed as mM of HOCl neutralized. The intra-assay CV was 5.87%. Fifteen samples were run once on two different plates, and the inter-assay CV was 9.79%.

Statistical analyses

11-ketotestosterone, CORT, aggression, d-ROM and OXY data were log transformed (ln x + 1), and T was square-root transformed prior to analysis to meet assumptions of parametric testing. We first investigated differences between the two species in hormones (CORT, T and 11-KT), aggressiveness or oxidative stress as a function of the three social contexts using ANOVAS. In these analyses, we also incorporated time of day of blood sampling (sampling took place from 9.00 to 15.15 h) as a covariate, because hormone levels may fluctuate diurnally (Oliveira *et al.*, 2001). We only report this effect when significant. Models were run in a stepwise backward manner, sequentially removing the nonsignificant (interaction) effects using a threshold of $P = 0.1$. We verified normality by examining residual plots and tested for homoscedasticity by applying Levene's test of homogeneity. T and 11-KT are highly linearly correlated (Pearson, $r = 0.839$, $P < 0.001$). Due to this collinearity, it is difficult to assess the independent effects of T and 11-KT. We therefore ran separate models using either 11-KT or T as a covariate. In the final models, we also tested for the effect of body mass difference between the focal male and the stimulus male; this had no effect on the findings (Ps > 0.2).

We corrected for multiple testing using the algorithm developed by Benjamini & Hochberg (1995). We set the false discovery rate (FDR) at $\alpha = 0.05$. In our study, three 'families' of hypotheses can be distinguished where FDR can be applied: those concerning species differences in aggressiveness ($n = 4$ tests, all P values ≥0.038 not significant after FDR control), those concerning a hormonal response upon a challenge ($n = 3$ tests, all P values ≥0.034 not significant after FDR control), those concerning the relationship between hormones and aggression ($n = 16$ tests, all P values ≥0.00625 not significant after FDR control) and those concerning the relationship between oxidative stress and hormones ($n = 4$ tests, all P values ≥0.0125 not significant after FDR control). Note that sample sizes (see result section) vary between the different measures because in some cases, there was insufficient volume of blood for all five different assays.

Experiment 2. Immune function

Experimental design

We used a total of 49 fish (not used in Experiment 1; blue fish: body mass, 24.8 ± 1.2 g mean ± SE, standard length, 94.1 ± 1.4, $n = 21$; red fish: body mass, 22.0 ± 0.7 g, standard length, 90.6 ± 0.9, $n = 28$). On Day 1, males were weighed and transferred to individual compartments with visual and chemical access to a neighbouring male of the same colour and similar body mass behind a perforated transparent partition to stimulate territoriality and development of nuptial colouration. The housing condition thus was identical to the *continuous social treatment* in the previous experiment. We only used this housing condition because it was in this treatment that we found the largest difference between species in oxidative stress (see below). Oxidative stress may affect immune function (Von Schantz *et al.*, 1999), and we therefore expected the most pronounced species difference on immune function in this treatment.

On Day 9 or 10, males were injected with a suspension of sheep red blood cells (SRBC; Harlan Inc., Horst, The Netherlands). For details, we refer to Dijkstra *et al.* (2007). After 13 days (on day 22 or 23), the estimated peak of antibody production (Dijkstra *et al.*, 2007), we collected 20–100 μL blood from the caudal vein with a 1-mL syringe to assess immune responses. The blood was mixed with a drop of heparin (<2 μL), and we then kept some of this blood for white blood cell characterization (see below). The remainder was centrifuged at 13 000 rpm for 10 min; blood plasma was then separated and stored at −20 °C for use in the agglutination assay.

Characterization of leucocytes

Changes in the humoral immune response, for instance via lower agglutination titres against SRBC, can be compensated through changes in the cell-mediated

immune response, as shown by, e.g. increased T cell proliferation (Kreukniet *et al.*, 1994). For this reason, we not only analysed the humoral immune response but included, using flow cytometry, an analysis of the blood leucocyte population. The majority of the peripheral blood leucocytes (PBL) consist of (B and T) lymphocytes, granulocytes and monocytes. B lymphocytes play a large role in the humoral immune response, whereas T lymphocytes are important in the cell-mediated immune response, granulocytes are involved in phagocytosis, and monocytes are involved in phagocytosis and antigen presentation (Iwama & Nakanishi, 1996). The relative abundance of large, activated lymphocytes can be estimated using a flow cytometer (Forlenza *et al.*, 2008), allowing us to test whether the two species differ in this measure of immune state.

Following blood collection (and mixing with a drop of heparin as mentioned above), we mixed 7.5 μL of the blood with 300 μL sterile heparinized cell culture medium (RPMI1640, Cambrex Bio Science Verviers, Belgium) and placed it on ice. Within 6 h of blood collection, total leucocytes were isolated by layering heparinized blood on a 1.0838 g mL^{-1} Percoll (Amersham, Biosciences, Roosendaal, The Netherlands) density gradient and subsequent centrifugation for 25 min at 2000 rpm. Leucocytes at the interface between cell culture medium and Percoll were collected and washed once in RPMI medium. Finally, cells were re-suspended in 300 μL of RPMI medium containing 1 μg mL^{-1} propidium iodide, to exclude necrotic cells. Forward scatter (FSC, corresponding to cell size) and side scatter (SSC, corresponding to cell complexity) characteristics of 10 000 events were acquired in linear mode, and fluorescence intensities were recorded on a log scale using a Beckman Coulter Epics XL-MCL flow cytometer. A gate was set to differentiate between cells with both low FSC and low SSC (small lymphocytes) and other cells (large lymphocytes, monocytes). This allowed us to estimate the relative abundance of large lymphocytes, that is, the proportion of large lymphocytes relative to the total number of lymphocytes. Note that monocytes were also included as they are difficult to distinguish from large lymphocytes by size and complexity alone. Because we were mainly interested in changes in lymphocyte morphology, granulocytes were excluded by gating.

Agglutination assay

Sheep red blood cell antibody concentrations in 20 μL of plasma were estimated in a standard agglutination titration assay (Hudson & Hay, 1989). For details, see Dijkstra *et al.* (2007). Titres were scored visually as the highest two-fold dilution of plasma showing agglutination. Preimmunization blood samples for the agglutination test were not taken. Commonly, antibody titre against SRBC is zero for unimmunized *Pundamilia* cichlids injected with saline only (n = 11, Dijkstra *et al.*, 2007, n = 4 this study).

Statistical analyses

Proportions (relative abundance of large lymphocytes) were arcsine-square root transformed to meet assumptions of parametric testing. To investigate differences between species in either antibody production or the relative abundance of large lymphocytes, we used ANOVAS or t-tests. Note that sample sizes vary between antibody response and white blood cell characterization, because when only a small volume of blood was available, we gave priority to the agglutination test. Time of sampling had no effect on the findings (results not shown). Also, the body mass difference between the focal male and the stimulus male did not affect the results (not shown). Statistical analyses for both experiments were carried out with SPSS 12.0.1 (Armonk, NY, USA). Significance tests were two-tailed with $\alpha = 0.05$. We report means ± standard error.

Results

Experiment 1. Oxidative stress, aggressiveness and hormone profiles

Aggressiveness

The difference between red and blue males in aggressiveness varied according to the two different social treatments (ANOVA, species × social treatment, display rate: $F_{1,33} = 5.80$, $P = 0.02$; attack rate: $F_{1,33} = 4.35$, $P = 0.045$). We therefore analysed the data for each treatment separately.

As expected, red males were significantly more aggressive than blue males in the continuous social treatment in both display rate (Fig. 1, $F_{1,20} = 5.52$, $P = 0.03$) and attack rate ($F_{1,20} = 6.39$, $P = 0.02$). In the intruder treatment, red males were also more aggressive in terms of display rate, but not attack rate (display rate: $F_{1,14} = 18.84$, $P = 0.001$; attack rate: $F_{1,14} = 0.26$, $P = 0.62$). In the final model, the time of day appeared to be predictive of display rates (but not attack rates), as aggression towards the intruder male declined over the course of the day (ANOVAS, effect of time of day on display rate: $F_{1,14} = 4.67$, $P = 0.049$; attack rate: $F_{1,14} = 3.31$, $P = 0.091$). This effect of sampling time was independent of species (interaction terms all $P > 0.1$).

Steroid Hormones

In this analysis, we also included the isolated males. There was no effect of species on T or 11-KT levels, contrary to our expectation (Fig. 2; ANOVAS, species effect on T: $F_{1,50} = 1.24$, $P = 0.27$; 11-KT: $F_{1,44} = 1.63$, $P = 0.21$; Treatment and times of day interaction retained in the model, see below). There was also no effect of species on CORT ($F_{1,48} = 1.03$, $P = 0.32$; treatment retained in the model, see below). To determine whether the sample sizes in our experiment were too small to detect significant differences in hormone values

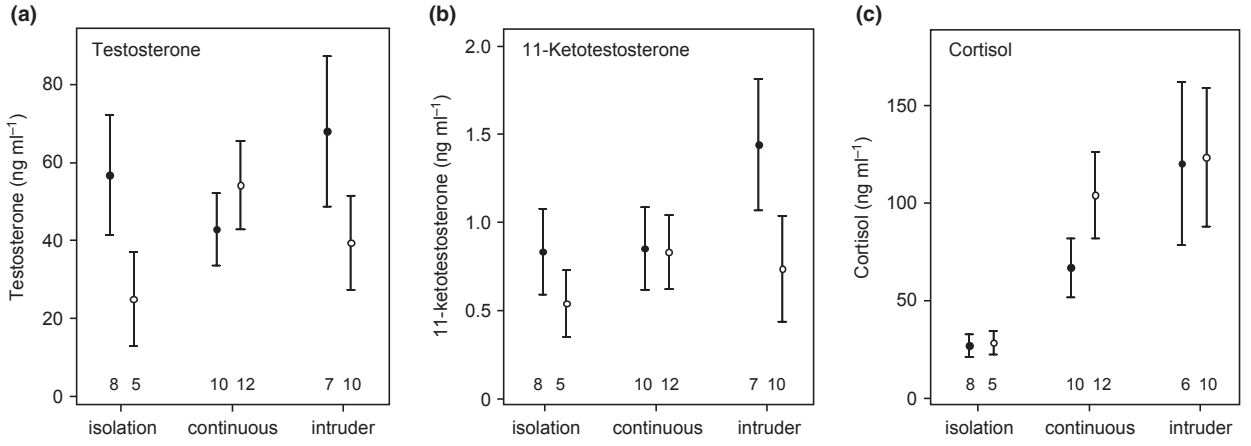

Fig. 2 Circulating levels of testosterone (a), 11-ketotestosterone (b) and cortisol (c) in ng mL^{-1} in the three different treatments (isolation, continuous social and intruder). Note that there are no significant differences between red (●) and blue males (○). Sample sizes are indicated at the bottom of each panel. Shown are the mean ± SE.

depending on social treatment and/or species, we performed a statistical power analysis (Cohen, 1988). We found that the power was 100% when the expected effect size was based on the display rate; and 57% (based on the average of individual power estimates for the three hormones), when based on the attack rate. Combining these two power estimates yielded an average power of 78%, which is very close to what is generally considered sufficient (> 80%, Cohen, 1988).

To our surprise, housing treatment did not have a significant effect on T ($F_{2,48} = 0.18$, $P = 0.84$). However, for 11-KT, we found a significant interaction effect between time of sampling and housing treatment ($F_{2,44} = 4.40$, $P = 0.018$). Early in the day, 11-KT was elevated in the intruder treatment compared with the other two treatments, but this hormonal response disappeared over the course of the day. Housing had a highly significant effect on CORT levels with higher levels in the social treatments ($F_{1,48} = 8.00$, $P = 0.001$). None of the interaction terms that included treatment and/or species were significant (all $P > 0.2$). We also re-ran the analysis focusing on the social treatments only, but results remained the same.

Steroid hormones and aggression

We tested whether hormone levels and aggressive behaviour are related while controlling for the role of species, using ANOVAS with either display rate or attack rate as response variable. In the starting model, we entered two hormones (either T and CORT or 11-KT and CORT), species and all two-way interaction effects. To facilitate interpretation, we analysed the two social treatments separately.

In the continuous social treatment, T levels were positively associated with attack rate (ANOVA, T: $F_{1,19} = 6.87$, $P = 0.02$), but not with display rate ($F_{1,19} = 2.45$, $P = 0.13$). Attack rate was positively and significantly predicted by 11KT levels ($F_{1,19} = 11.24$, $P = 0.003$, species: $F_{1,19} = 9.27$, $P = 0.007$). An interaction between 11-KT and CORT had a significant effect (after FDR control) on display rate ($F_{1,18} = 11.12$, $P = 0.004$), due to 11-KT and CORT showing nonsignificant, though contrasting relationships with display rate (11-KT, Pearson, $r = 0.31$, $P = 0.16$; CORT: $r = -0.16$, $P = 0.47$, $n = 22$). Attack rate was not related to CORT levels (starting model with T, effect of CORT: $F_{1,18} = 0.95$, $P = 0.34$; starting model with 11-KT, effect of CORT: $F_{1,18} = 1.09$, d.f. = 1, 18, $P = 0.31$). As indicated earlier, there was a significant interaction between CORT and 11-KT on display rate. However, there was no interaction effect between CORT and T ($F_{1,16} = 0.57$, $P = 0.46$), nor was there an effect of CORT on display rate ($F_{1,18} = 0.45$, $P = 0.51$).

In the intruder treatment, attack rate was not related to T or 11-KT (ANOVA, T: $F_{1,12} = 1.56$, $P = 0.24$; 11-KT: $F_{1,11} = 0.27$, $P = 0.61$). Display rate was significantly correlated with 11-KT ($F_{1,12} = 6.53$, $P = 0.03$; species: $F_{1,12} = 8.97$, $P = 0.011$) and showed a similar though nonsignificant trend with T ($F_{1,14} = 3.04$, $P = 0.10$; species: $F_{1,14} = 12.20$, $P = 0.004$). CORT had no significant effect on attack rate when the starting model contained T ($F_{1,14} = 0.019$, $P = 0.89$). When the starting model contained 11-KT, the interaction term between species and CORT was retained in the model as a nonsignificant predictor of attack rate ($F_{1,12} = 4.13$, $P = 0.07$). CORT had no significant effect on display rate (starting model with T, $F_{1,13} = 0.64$, $P = 0.44$; starting model with 11-KT, $F_{1,12} = 3.41$, $P = 0.09$). After FDR control, none of the interaction terms that included species were significant, indicating that the direction and angle of the correlations are the same for red and blue males, although sometimes at a different absolute level (effect of species).

Oxidative stress

Overall, neither ROM levels (d-ROM) nor antioxidant capacity (OXY) differed significantly between the two species (Table 1, ANOVAS comparing species, d-ROM: $F_{1,42} = 0.64$, $P = 0.43$; OXY: $F_{1,42} = 1.06$, $P = 0.31$; in both models, housing treatment and the interaction term between treatment and species were removed from the model, $P > 0.12$). However, the level of oxidative stress (the ratio of d-ROM to OXY) was not only significantly different across treatments ($F_{2,40} = 3.91$, $P = 0.028$), but also significantly higher in red males than in blue males (Fig. 3, $F_{1,40}$ $F = 5.30$, $P = 0.027$), consistent with our prediction. Interestingly, the difference in oxidative stress between species was only significant in the two social treatments (independent *t*-test in which the continuous social treatment and intruder treatment are combined: $t_{31} = 2.46$, $P = 0.02$; isolation treatment: $t_9 = 0.03$, $P = 0.97$), suggesting that the difference in oxidative stress between the species is dependent on the social context. This is also supported by a marginally nonsignificant treatment effect on the level of oxidative stress in red males only (ANOVAS, red males: treatment effect $F_{2,18}$ $F = 3.41$, $P = 0.055$; blue males: treatment effect $F_{2,20} = 0.61$, $P = 0.55$), although the interaction term between species and treatment was not significant ($F_{2,38}$ $F = 1.66$, $P = 0.21$).

We also explored possible relationships between oxidative stress and hormone profiles, using an ANOVA with oxidative stress as response variable, housing condition and species as explanatory variable, and measures of T and CORT, or 11-KT and CORT as covariates. When the starting model contained T, T was as a significant negative predictor of oxidative stress ($F_{1,38}$ $F = 13.56$, $P = 0.001$), whereas CORT was retained as a nonsignificant effect in the final model ($F_{1,38}$ $F = 3.64$, $P = 0.06$). Other factors retained in the model were species ($F_{1,38}$ $F = 12.31$, $P = 0.001$) and treatment ($F_{2,18}$ $F = 3.88$, $P = 0.03$). When the starting model contained 11-KT, there was an interaction between species and 11-KT ($F_{1,36} = 4.30$, $P = 0.045$) and between species and CORT ($F_{1,36} = 6.66$, $P = 0.014$). Treatment was also retained in the model ($F_{2,36} = 3.41$, $P = 0.044$), but none of these effects remained significant after FDR control.

Table 1 The d-reactive oxygen metabolite (ROM) (Carratelli units, see Costantini *et al.*, 2006) and OXY (μmol of HClO mL^{-1}) values for red and blue fish in the three different treatments (isolation, continuous social and intruder). Shown are the mean and SE.

	d-ROM		OXY	
Treatment	Red males	Blue males	Red males	Blue males
Isolation	8.8 ± 1.1	7.5 ± 0.8	232.8 ± 19.6	200 ± 6.2
Continuous	11.35 ± 1.3	9.1 ± 0.4	187 ± 15.5	216 ± 8.2
Intruder	10.3 ± 1.5	10.1 ± 0.8	212 ± 21.6	228 ± 12.2

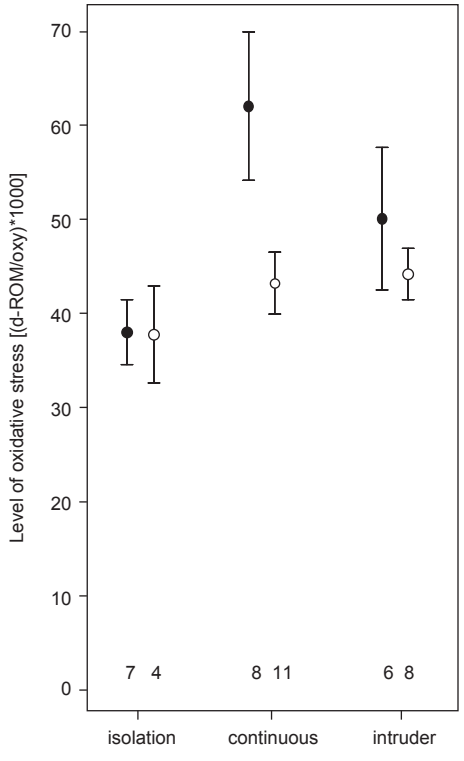

Fig. 3 Oxidative stress (ratio between d-reactive oxygen metabolite and OXY) was higher for red (●) than for blue males (○), but only in the social treatments. Sample sizes are indicated at the bottom. Shown are the mean ± SE.

Experiment 2. Immune function

The mean antibody response of blue males was 7.08 (±0.52, $n = 21$) and of red males 4.39 (±0.57, $n = 28$). This difference was highly significant (ANOVA, $F_{1,47} = 12.18$, $P = 0.001$). The relative abundance of large lymphocytes did not differ between red and blue males (blue fish: 0.38 ± 0.05 mean ± SE, $n = 21$; red fish: 0.37 ± 0.04, $n = 27$, ANOVA, $F_{1,46} = 0.15$, $P = 0.70$).

Discussion

In this study, we asked whether red males (*P. nyererei*) bear a physiological cost that might constitute a trade-off with their documented social dominance advantage over blue males (*P. pundamilia*). First, we confirmed that red males were more aggressive than blue males, consistent with earlier findings (Dijkstra *et al.*, 2009a). We also found that red males, when housed in a territorial context, indeed experienced more oxidative stress and produced fewer antibodies than blue males. We first discuss several hypotheses that might causally underlie the physiological differences between red and blue males. We then explore implications of these differences for understanding the coexistence and evolution of red and blue *Pundamilia* males.

Species differ in oxidative stress and immune function

We found that only red males and not blue males experienced more oxidative stress when they were housed in a social condition. Social stimulation leads to territoriality, which is not only associated with brighter colouration, but also increased energy expenditure (Ros *et al.*, 2006a), higher androgen levels (Dijkstra *et al.*, 2007), testes maturation (Fraley & Fernald, 1982) and a suite of other neurophysiological characteristics (Trainor & Hofmann, 2006). Our findings thus suggest that red males face more costs or constraints than blue males when up-regulating the reproductive axis.

There are at least two mechanisms that could explain the relationship between colour, oxidative stress and territorial status. First, social stimulation triggers a gradual increase in the intensity of the nuptial dress in red males (up to three-fold, over the course of days rather than hours), likely involving incorporation of additional carotenoids into the skin (see Dijkstra *et al.*, 2007). Because the nuptial dress of red males contains far more carotenoids than that of blue males (Maan *et al.*, 2006), it is possible that red males in a social situation allocate carotenoids towards their nuptial dress and away from antioxidant function, whereas blue males do not undergo this reallocation. Consistent with such an explanation, red males in our study did indeed suffer from higher oxidative stress, but only in a social situation. It is likely that a trade-off in carotenoid allocation may only apply when carotenoids are limiting (Pike *et al.*, 2007), which may be the case in the field. However, fish in our study were fed with normal cichlid flake food containing plenty of carotenoids. Future experiments should examine the carotenoid trade-off hypothesis in more detail by limiting carotenoid intake (Clotfelter *et al.*, 2007; Pike *et al.*, 2007). Alternatively, it is possible that the species difference in oxidative stress levels is caused by differences in metabolism resulting from the higher level of aggression of red males compared with blue males (Careau *et al.*, 2008). Because aggression is metabolically costly, it could be hypothesized that differences in metabolic rate associated with activity during territorial behaviour were responsible for the observed difference in oxidative stress between species (Leeuwenburgh & Heinecke, 2001; Ros *et al.*, 2006a).

We found that red males produced fewer antibodies against a foreign antigen than blue males, whereas the relative abundance of large activated lymphocytes was not different between the two species. This suggests that the compromised state of the humoral immune response in red males was not compensated for by a stimulated state of the cell-mediated immune response, as has been found in chickens (Kreukniet *et al.*, 1994). A high humoral immune response to SRBC has been associated with high humoral immune responses to a variety of antigens (Parmentier *et al.*, 2001). In addition to the humoral immune response, we also analysed blood leucocyte populations of red and blue males to test whether changes in the humoral immune response – for instance via lower agglutination titres against SRBC – can be compensated through changes in the cell-mediated immune response (Kreukniet *et al.*, 1994). Because of the complexity of the immune system, we are cautious with the interpretation of our findings, though it appears that blue males probably have a more responsive humoral immune system than red males.

The difference in immune response between red and blue males is consistent with the literature. First, it has previously been shown that carotenoid-dependent sexual traits can be traded off against immune function because carotenoids enhance immune function (e.g. Alonso–Alvarez *et al.*, 2004; but see Fitze *et al.*, 2007), including in fish (Grether *et al.*, 2004; Clotfelter *et al.*, 2007). For example, we have previously shown that expressing red colouration imposes an immunological cost in red *Pundamilia* males (Dijkstra *et al.*, 2007). Consistent with this allocation trade-off, we observed a lower immune response in red males, which have far more carotenoids in their nuptial dress than blue males (Maan *et al.*, 2006). Secondly, oxidative stress is known to affect a variety of bodily functions including immunity (Von Schantz *et al.*, 1999). Because the housing condition was identical to the continuous social treatment in Experiment 1 where we found that red males experienced higher oxidative stress levels than blue males, it is plausible that oxidative stress compromised the antibody response in red males more than in blue males.

The role of steroid hormones

Androgens have been suggested to suppress the immune system (Folstad & Karter, 1992) and increase oxidative stress (Von Schantz *et al.*, 1999). 11-ketotestosterone has been shown to have suppressive effects on several measures of immunity and to increase oxidative stress (Kurtz *et al.*, 2007; see also Ros *et al.*, 2006b). Additionally, it is well known that CORT can modulate immune activity (for reviews for fish, see Wendelaar Bonga, 1997). As a first assessment of whether the differences in antibody response and oxidative stress levels between the two species could be mediated by these steroids, we investigated whether red and blue males have different hormone level profiles. We found that 11-KT, but not testosterone (T) levels, correlated positively with aggressiveness, which is consistent with the suggestion that 11-KT is the behaviourally relevant androgen, rather than T (Kime, 1993; but see Trainor & Hofmann, 2006; Greenwood *et al.*, 2008). CORT was not related to aggressiveness. Unexpectedly, we failed to detect higher T, 11-KT or CORT levels in the more aggressive red males. Typically, animals show a strong hormonal response to a social challenge (Wingfield *et al.*, 1990; Hirschenhauser *et al.*, 2004), and following this, we

detected elevated steroid levels in 11-KT in the intruder treatment, with this response attenuating over de course of the day. This time effect is consistent with previous reports (Oliveira *et al.*, 2001).

Importantly, the patterns in hormone variation were not species-specific. Pronounced endocrine variation between closely related species has been reported in several taxa (Hirschenhauser *et al.*, 2004; Owen–Ashley & Butler, 2004), yet in other species, the assumed proximate link between steroids and aggression levels is less clear (Ball & Balthazart, 2008). Based on our results, we suggest that the species differences in aggressiveness are not mediated by differences in circulating androgen levels, but that they might be mediated at various levels of the steroid signalling cascade (e.g. steroid receptor expression, steroid binding proteins regulating steroid availability and enzymatic actions in the brain; see e.g. Jennings *et al.*, 2000). In addition, neuropeptide hormones such as arginine vasotocin mediate behavioural differences across vertebrates (Goodson & Bass, 2001; Greenwood *et al.*, 2008).

We found that T was negatively correlated with oxidative stress, but it is difficult to say whether this is in agreement with the notion that androgens induce oxidative stress or not because oxidative stress can also influence androgen levels (Von Schantz *et al.*, 1999). Because the two species did not differ in circulating levels of any of the three steroids, it appears more likely that the observed species difference in oxidative stress and immunity was linked to species-specific pigmentation allocation strategies and/or the metabolic costs of aggressiveness, as suggested above (Ros *et al.*, 2006a; Clotfelter *et al.*, 2007). Alternatively, it is possible that red and blue males differ in sensitivity to androgens such that similar circulating androgen levels have differential effects on oxidative stress and immunity. Future experiments, employing hormonal perturbations or quantification of androgen receptor expression in different target tissues, will shed more light on these possibilities.

Coexistence of red and blue in nature

Previous studies showed that red males experience a social dominance advantage in dyadic combat with blue males (Dijkstra *et al.*, 2005). We hypothesized that a lower immune responsiveness and/or higher oxidative stress in red males has negative fitness consequences and as a result may counterbalance their advantage in aggressive interactions, stabilizing the coexistence of red and blue in our study population. Although we lack direct fitness estimates, we found some evidence for this hypothesis in this study. The physiological costs to red males may help explain why red *Pundamilia* populations appear to always occur sympatrically with at least one blue species, whereas entirely blue populations are not uncommon (Seehausen & Van Alphen, 1999). In addition, we recently found that the social dominance advantage of red males is frequency-dependent, possibly due to increased red-red conflict in red-biased communities attenuating the dominance advantage of red males over blue males (Dijkstra *et al.* 2010). It is tempting to speculate that the physiological costs to red males are not only dependent on social status, but also on their relative abundance in the population (for an example of frequency-dependent physiological performance, see Pryke *et al.*, 2007). Other factors may also affect their coexistence, including ecological differentiation, ambient light properties, predation risk (Fig. 4 arrow A) and mate choice (Fig. 4, arrow C) (reviewed in Seehausen, 2009). Factors that promote long-lasting coexistence of species are also important for stabilizing the process of speciation, increasing the time window for divergent and disruptive selection to act (van Doorn *et al.*, 2004). A challenge will be to identify how social, ecological and physiological factors jointly influence and maintain species diversity in haplochromines.

In Lake Victoria, several separate *Pundamilia* populations exist where males display red, blue or intermediate colouration along a gradient of water clarity (described as a 'speciation transect', Seehausen, 2009). The focus of

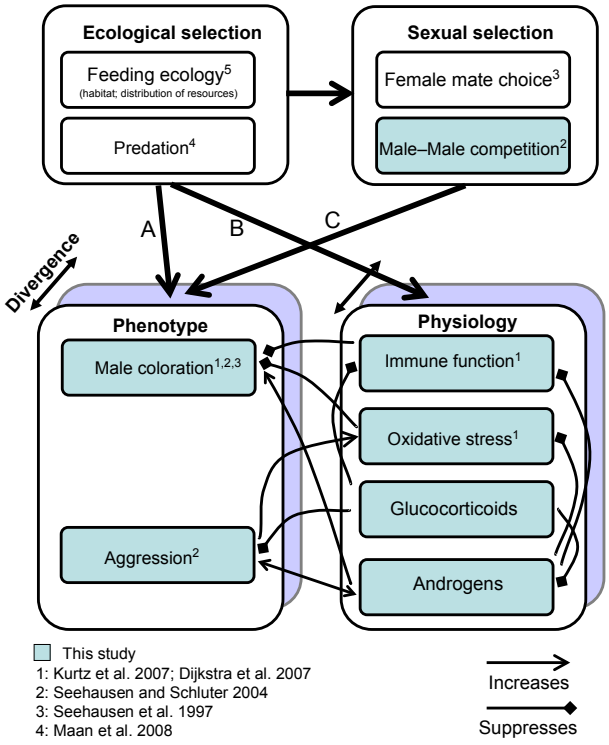

Fig. 4 Simplified representation of our conceptual framework showing how physiological attributes are fundamental to our understanding of how ecological and sexual selection can drive differentiation in male phenotype. The blue boxes denote the factors that are most relevant in the current study. Note that for the sake of clarity, only the most obvious relationships are indicated, and the direction of the effect may be ambiguous.

the present study was on the Kissenda Island species where red and blue populations form a frequently hybridizing incipient species pair (Magalhaes *et al.*, 2009). We predict, however, that the relationship between colour and physiology applies to the entire speciation transect, and possibly many other cases, given the abundance of red-blue sister species and polymorphisms among Lake Victoria cichlid fish (Seehausen & Van Alphen, 1999). Consistent with this prediction, red and blue *Pundamilia* males from Python Island showed the same humoral response pattern as we report here (Dijkstra, van der Sluijs, unpublished results). A study by Seehausen *et al.* (2008) compared red and blue phenotypes along the 'speciation transect' and found strong correlations between ambient light colour, male nuptial colour, visual pigments and female mating preference for male nuptial colour, which provided evidence for speciation through sensory drive.

Along the 'speciation transect', red males become increasingly more restricted to greater depth with increasing water transparency (or rather, decreasing light slope, see Seehausen *et al.*, 2008). We studied red and blue males from Kissenda Island where red males also occur at greater depths than blue males, though the two incipient species show substantial overlap in vertical distribution (Seehausen *et al.*, 2008). Consequently, divergent adaptation might have contributed to the observed physiological differences, in particular in immune function (Fig. 4, arrow B; see e.g. Blais *et al.*, 2007; Maan *et al.*, 2008). Interestingly, disruptive ecological selection favours the evolution of sexual preferences for specific ornaments (such as different colour types) that signal local adaptation (Van Doorn *et al.*, 2009), and in the case of *Pundamilia*, adaptation to the local light regime (Seehausen *et al.*, 2008). Thus, it is possible that species divergence in immunity due to local adaptation might be facilitated or constrained by colour-specific physiological trade-offs between sexual signalling and health maintenance.

In the framework of evolutionary diversification, it is important to distinguish between several unique roles of physiology. First, as illustrated in Fig. 4, selection on one trait is likely to impact the expression of other traits due to pleiotropy and/or allocation trade-offs, a phenomenon that is well recognized in life-history evolution (e.g. Folstad & Karter, 1992; Ardia *et al.*, 2011). Because of these interrelationships between traits, divergent or disruptive selection on multiple traits cannot be viewed separately. Secondly, as suggested by our results, differences in trade-offs between sexual signalling and physiological performance may stabilize the coexistence of diverging phenotypes through balancing selection. Finally, physiological trade-offs can be under negative frequency-dependent selection (Pryke *et al.*, 2007). It will be interesting to explore how physiological trade-offs influence the scope and conditions for adaptive speciation using theoretical approaches.

Sexual selection has been implicated in the dramatic colour diversification of East African cichlid fish (e.g. Kocher, 2004; Seehausen & Schluter, 2004), but the physiological underpinnings of this process have received very little attention. In the present study, we have shown that divergence in colouration and agonistic behaviour in two incipient species of haplochromine cichlids has been accompanied by differentiation in immune function and oxidative stress, but not in hormone profile. In addition, it appears that the two species differ in the trade-off between gaining territorial status and oxidative stress. Asymmetric aggression between conspecific colour morphs and sister species appears to be common in East African cichlid fishes (e.g. Korzan *et al.*, 2008; Dijkstra *et al.*, 2009b). Correlated divergence in colour, behaviour and physiology might be widespread in the dramatically diverse cichlid radiations in East African lakes and may play a crucial role in the remarkably rapid speciation of these fish.

Acknowledgments

We thank Roelie Veenstra–Wiegman, Sjoerd Veenstra, Monique Huizinga and Sara Schaafsma for their assistance with fish care. We are very grateful to Anja Taverne–Thiele, Bernd Riedstra and Simon Verhulst for help with the immune assay, and Winnie Boner for help with the oxidative stress assays. We thank Celeste Kidd and Lin Huffman for technical assistance with the hormone assays. Lauren O'Connell, Martine Maan, Anne Peters and Ole Seehausen provided useful comments on earlier versions of the manuscript. We thank Thomas Flatt and three anonymous reviewers for improving the manuscript. The research was supported by a Rubicon grant from the Dutch government, a Fisheries Society of the British Isles Research grant and an EU (International Outgoing Marie Curie fellowship) to P.D.D., and a NWO (SLW) grant to T.G.G. The research was carried out with an animal experiment licence (DEC 3137 and DEC 4335A) from Groningen University and complied with current laws in The Netherlands.

References

Alonso–Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. *et al.* 2004. An experimental test of the dose–dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. *Am. Nat.* **164**: 651–659.

Ardia, D.R., Parmentier, H.K. & Vogel, L.A. 2011. The role of constraints and limitation in driving individual variation in immune response. *Funct. Ecol.* **25**: 61–73.

Baerends, G.P. & Baerends–Van Roon, J.M. 1950. An introduction to the study of the ethology of cichlid fishes. *Behav. Suppl.* **1**: 233–366.

Bagni, M., Civitareale, C., Priori, A., Ballerini, A., Finoia, M., Brambilla, G. *et al.* 2007. Pre–slaughter crowding stress and killing procedures affecting quality and welfare in sea bass

(*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). *Aquaculture* **263**: 52–60.

Ball, G.F. & Balthazart, J. 2008. Individual variation and the endocrine regulation of behaviour and physiology in birds: a cellular/molecular perspective. *Phil. Trans. R. Soc. B* **363**: 1699–1710.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* **57**: 289–300.

Blais, J., Rico, C., Oosterhout, C., Cable, J. & Turner, G.F. 2007. MHC adaptive divergence between closely related and sympatric African Cichlids. *PLoS ONE* **2**: e734.

Blount, J.D. 2004. Carotenoids and life–history evolution in animals. *Arch. Biochem. Biophys.* **430**: 10–15.

Careau, V., Thomas, D., Humphries, M.M. & Réale, D. 2008. Energy Metabolism and Animal Personality. *Oikos* **117**: 641–653.

Clotfelter, E.D., Ardia, D.R. & McGraw, K.J. 2007. Red fish, blue fish: trade–offs between pigmentation and immunity in *Betta splendens*. *Behav. Ecol.* **18**: 1139–1145.

Cohen, J. 1988. *Statistical Power Analysis for the Behavioral Sciences*, 2nd edn. Lawrence Erlbaum Associates, New York.

Costantini, D., Casagrande, S., De Filippis, S., Brambilla, G., Fanfani, A., Tagliavini, J. *et al.* 2006. Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). *J. Comp. Physiol. B.* **176**: 329–337.

Dijkstra, P.D., Seehausen, O. & Groothuis, T.G.G. 2005. Direct male–male competition can facilitate invasion of new colour types in Lake Victoria cichlids. *Behav. Ecol. Sociobiol.* **58**: 136–143.

Dijkstra, P.D., Seehausen, O., Gricar, B.L.A., Maan, M.E. & Groothuis, T.G.G. 2006. Can male–male competition stabilize speciation? A test in Lake Victoria haplochromine cichlid fish. *Behav. Ecol. Sociobiol.* **59**: 704–713.

Dijkstra, P.D., Hekman, R., Schulz, R.W. & Groothuis, T.G.G. 2007. Social stimulation, nuptial coloration, androgens, and immunocompetence in a sexual dimorphic cichlid fish. *Behav. Ecol. Sociobiol.* **61**: 599–609.

Dijkstra, P.D., van der Zee, E.M. & Groothuis, T.G.G. 2008. Territory quality affects female preference in a Lake Victoria cichlid fish. *Behav. Ecol. Sociobiol.* **62**: 747–755.

Dijkstra, P.D., Hemelrijk, C.K., Seehausen, O. & Groothuis, T.G.G. 2009a. Colour polymorphism and intrasexual selection in assemblages of cichlid fish. *Behav. Ecol.* **20**: 138144.

Dijkstra, P.D., van Dijk, S., Groothuis, T.G.G., Pierotti, M.E.R. & Seehausen, O. 2009b. Behavioral dominance between female color morphs of a Lake Victoria cichlid fish. *Behav. Ecol.* **20**: 593–600.

Dijkstra, P.D., Lindström, J., Metcalfe, N.B., Hemelrijk, C.K., Brendel, M., Seehausen, O. *et al.* 2010. Frequency-dependent social dominance in a color polymorphic cichlid fish. *Evolution* **64**: 2797–2807.

van Doorn, G.S., Diekmann, U. & Weissing, F.J. 2004. Sympatric speciation by sexual selection, a critical re-evaluation. *Am. Nat.* **163**: 709–725.

Dowling, D.K. & Simmons, L.W. 2009. ROS as universal constraints in life-history evolution. *Proc. R. Soc. Lond. B* **276**: 1737–1745.

Ducrest, A.-L., Keller, L. & Roulin, A. 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends Ecol. Evol.* **23**: 502–510.

Finkel, T. & Holbrook, N.J. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* **408**: 239–247.

Fitze, P.S., Tschirren, B., Gasparini, J. & Richner, H. 2007. Carotenoid–based plumage colors and immune function: Is there a trade–off for rare carotenoids? *Am. Nat.* **169**: S137–S144.

Folstad, I. & Karter, A.J. 1992. Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* **139**: 603–622.

Forlenza, M., Taverne–Thiele, J.J., Kachamakova, N., Scharsack, J.P., Rombout, J.H.W.M. & Wiegertjes, G.F. 2008. Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host-parasite animal model. *Mol. Immunol.* **45**: 3178–3189.

Fraley, N.B. & Fernald, R.D. 1982. Social control of developmental rate in the African cichlid fish, *Haplochromis burtoni*. *Z. Tierpsychol.* **60**: 66–82.

Genner, M.J. & Turner, G.F. 2005. The mbuna cichlids of Lake Malawi: a model for rapid speciation and adaptive radiation. *Fish Fish.* **6**: 1–34.

Goodson, J.L. & Bass, A.H. 2001. Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. *Brain Res. Rev.* **35**: 246–265.

Gray, S.M. & McKinnon, J.S. 2007. Colour polymorphism and implications for speciation. *Trends Ecol. Evol.* **22**: 71–79.

Greenwood, A.K., Wark, A.R., Fernald, R.D. & Hofmann, H.A. 2008. Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. *Proc. R. Soc. Lond. B* **275**: 2393–2402.

Grether, G.F., Kasahara, S., Kolluru, G.R. & Cooper, E.L. 2004. Sex-specific effects of carotenoid intake on the immunological response to allograft in guppies (*Poecilia reticulata*). *Proc. R. Soc. Lond. B* **271**: 45–49.

Griffith, S.C., Parker, T.H. & Olson, V.A. 2006. Melanin – versus carotenoid-based sexual signals: is the difference really so black and red? *Anim. Behav.* **71**: 749–763.

Hill, G.E. 1999. Is there an immunological cost to carotenoid-based ornamental colouration. *Am. Nat.* **154**: 589–595.

Hirschenhauser, K., Taborsky, M., Oliveira, T., Canario, A.V.M. & Oliveira, R.F. 2004. A test of the 'challenge hypothesis' in cichlid fish: simulated partner and territory intruder experiments. *Anim. Behav.* **68**: 541–550.

Hofmann, H.A. 2003. Functional genomics of neural and behavioral plasticity. *J. Neurobiol.* **54**: 272–282.

Hofmann, H.A., Benson, M.E. & Fernald, R.D. 1999. Social status regulates growth rate: consequences for life-history strategies. *Proc. Natl Acad. Sci. USA* **95**: 14171–14176.

Hudson, L. & Hay, F.C. 1989. *Practical Immunology*. Blackwell, Oxford.

Iwama, G. & Nakanishi, T. 1996. *The Fish Immune System: Organism, Pathogen, and Environment*. Academic Press, San Diego.

Jennings, D.H., Moore, M.C., Knapp, R., Matthews, L. & Orchinik, M. 2000. Plasma steroid-binding globulin mediation of differences in stress reactivity in alternative male phenotypes in tree lizards, *Urosaurus ornatus*. *Gen. Comp. Endocrinol.* **120**: 289–299.

Kidd, C., Kidd, M.R. & Hofmann, H.A. 2010. Measuring multiple hormones from a single water sample using enzyme immunoassays. *Gen. Comp. Endocrinol.* **165**: 277–285.

Kime, D. 1993. 'Classical' and 'non-classical' reproductive steroids in fish. *Rev. Fish Biol. Fish.* **3**: 160–180.

Kocher, T.D. 2004. Adaptive evolution and explosive speciation: the cichlid fish model. *Nat. Genet.* **5**: 289–298.

Korzan, W.J., Robison, R.R., Zhao, S. & Fernald, R.D. 2008. Color change as a potential behavioral strategy. *Horm. Behav.* **54**: 463–470.

Kreukniet, M.B., Gianotten, N., Nieuwland, M.B. & Parmentier, H.K. 1994. *In vitro* T cell activity in two chicken lines divergently selected for antibody response to sheep erythrocytes. *Poult. Sci.* **73**: 336–340.

Kurtz, J., Kalbe, M., Langefors, A., Mayer, I., Milinski, M. & Hasselquist, D. 2007. An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. *Am. Nat.* **170**: 509–519.

Leeuwenburgh, C. & Heinecke, J.W. 2001. Oxidative stress and antioxidants in exercise. *Curr. Med. Chem.* **8**: 829–838.

Lozano, G.A. 1994. Carotenoids, parasites, and sexual selection. *Oikos* **70**: 309–311.

Maan, M.E., Seehausen, O., Soderberg, L., Johnson, L., Ripmeester, A.P., Mrosso, H.D.J. *et al.* 2004. Intraspecific sexual selection on a speciation trait, male colouration, in the Lake Victoria cichlid *Pundamilia nyererei*. *Proc. R. Soc. Lond. B* **271**: 2445–2452.

Maan, M.E., van der Spoel, M., Jimenez, P.W., van Alphen, J.J.M. & Seehausen, O. 2006. Fitness correlates of male coloration in a Lake Victoria cichlid fish: evidence for parasite-mediated sexual selection. *Behav. Ecol.* **17**: 691–699.

Maan, M.E., van Rooijen, A.M.C., van Alphen, J.J.M. & Seehausen, O. 2008. Parasite mediated sexual selection and species divergence in Lake Victoria cichlid fish. *Biol. J. Linn. Soc.*, **94**: 53–60.

Magalhaes, I.S., Mwaiko, S., Schneider, M.V. & Seehausen, O. 2009. Divergent selection and phenotypic plasticity during incipient speciation in Lake Victoria cichlid fish. *J. Evol. Biol.* **22**: 260–274.

Matsuo, M. & Kakato, T. 2000. The chemistry of reactive oxygen species and related free radicals. In: *Free Radical in Exercise and Aging* (Z. Rada'k, ed.), pp. 1–34. Human Kinetics, Champaign.

McGlothlin, J.W. & Ketterson, E.D. 2008. Hormone-mediated suites as adaptations and evolutionary constraints. *Phil. Trans. R. Soc. B* **363**: 1611–1620.

McGraw, K.J. 2005. Antioxidant function of many animal pigments: consistent health benefits of sexually selected colorants? *Anim. Behav.* **69**: 757–764.

McKinnon, J.S. & Pierotti, M.E.R. 2010. Color polymorphism and correlated characters: genetic mechanisms and evolution. *Mol. Ecol.* **19**: 5101–5125.

Metcalfe, N.B. & Alonso-Alvarez, C. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death *Funct. Ecology* **24**: 984–996.

Mills, S.C., Hazard, L., Lancaster, L., Mappes, T., Miles, D., Oksanen, T.A. *et al.* 2008. Gonadotropin hormone modulation of testosterone, immune function, performance, and behavioral trade-offs among male morphs of the lizard Uta stansburiana. *Am. Nat.* **3**: 339–357.

Monaghan, P., Metcalfe, N.B. & Torres, R. 2009. Oxidative stress as a mediator of life history trade–offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**: 75–92.

Nosil, P. & Harmon, L. 2009. Niche dimensionality and ecological speciation. In: *Speciation and Patterns of Diversity* (R. Butlin, J. Bridle & D. Schluter, eds), pp. 127–154. Cambridge University Press, Cambridge.

Oliveira, R.F., Lopes, M., Carneiro, L.A. & Canário, A.V.M. 2001. Watching fights raises fish hormone levels. *Nature* **409**: 475.

Owen–Ashley, N.T. & Butler, L.K. 2004. Androgens, interspecific competition and species replacement in hybridizing warblers. *Biol. Lett.* **271**: S498–S500.

Parmentier, H.K., Yousif Abuzeid, S., de Vries Reilingh, G., Nieuwland, M.G.B. & Graat, E.A.M. 2001. Immune responses and resistance to *Eimeria acervulina* of chickens divergently selected for antibody responses to sheep red blood cells. *Poult. Sci.* **80**: 894–900.

Pauers, M.J., Kapfer, J.M., Fendos, C.E. & Berg, C.S. 2008. Aggressive biases towards similarly coloured males in Lake Malawi cichlid fishes. *Biol. Lett.* **4**: 156–159.

Pike, T.W., Blount, J.D., Bjerkeng, B., Lindström, J. & Metcalfe, N.B. 2007. Carotenoids, oxidative stress and female mating preference for longer-lived males. *Proc. R. Soc. Lond. B* **274**: 1591–1596.

Pryke, S.R., Astheimer, L.B., Buttemer, W.A. & Griffith, S.C. 2007. Frequency–dependent physiological trade-offs between competing color morphs. *Biol. Lett.* **3**: 494–497.

Ros, A.F.H., Becker, K. & Oliveira, R.F. 2006a. Aggressive behaviour and energy metabolism in a cichlid fish, *Oreochromis mossambicus*. *Physiol. Behav.* **89**: 164–170.

Ros, A.F.H., Bouton, N., Santos, R.S. & Oliveira, R.F. 2006b. Alternative male reproductive tactics and the immunocompetence handicap in the Azorean rock-pool blenny, *Parablennius parvicornis*. *Proc. R. Soc. Lond. B* **273**: 901–909.

Salzburger, W. 2009. The interaction of sexually and naturally selected traits in the adaptive radiations of cichlid fishes. *Mol. Ecol.* **18**: 169–185.

Salzburger, W. & Meyer, A. 2004. The species flocks of East African cichlid fishes: recent advances in molecular phylogenetics and population genetics. *Naturwissenschaften*, **91**: 277–290.

Schluter, D. 2000. *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford.

Seehausen, O. 2006. African cichlid fish: a model system in adaptive radiation research. *Proc. R. Soc. Lond. B* **273**: 1987–1998.

Seehausen, O. 2009. The sequence of events along a ''speciation transect'' in the Lake Victoria cichlid fish *Pundamilia*. In: *Speciation and Patterns of Diversity* (R. Butlin, J. Bridle & D. Schluter, eds), pp. 155–176. Cambridge University Press, Cambridge.

Seehausen, O. & Schluter, D. 2004. Male-male competition and nuptial-colour displacement as a diversifying force in Lake Victoria cichlid fishes. *Proc. R. Soc. Lond. B* **271**: 1345–1353.

Seehausen, O. & Van Alphen, J.J.M. 1999. Can sympatric speciation by disruptive sexual selection explain rapid evolution of cichlid diversity in Lake Victoria? *Ecol. Lett.* **2**: 262–271.

Seehausen, O., van Alphen, J.J.M. & Witte, F. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science* **277**: 1808–1811.

Seehausen, O., Lippitsch, E., Bouton, N. & Zwennes, H. 1998. *Mbipi*, the rock-dwelling cichlids of Lake Victoria: description of three new genera and fifteen new species (*Teleostei*). *Ichthyol. Explor. Freshw.* **9**: 129–228.

Seehausen, O.Y., Terai, Y., Magalhaes, I.S., Carleton, K.L., Mrosso, H., Miyagi, R. *et al.* 2008. Speciation through sensory drive in cichlid fish. *Nature* **455**: 620–626.

Sinervo, B. & Calsbeek, R. 2006. The development, physiological, neural and genetic causes and consequences of frequency-dependent selection in the wild. *Ann. Rev. Ecol. Evol. Syst.* **37**: 581–610.

Soma, K.K., Scotti, M.A.L., Newman, A.E.M., Charlier, T.D. & Demas, G.E. 2008. Novel mechanisms for neuroendocrine regulation of aggression. *Front. Neuroendocrinol.* **29**: 476–489.

Tanganelli, I., Ciccoli, L., Tansi, R., Borgogni, P., Rossi, V., Gistri, M. *et al.* 2000. Markers of oxidative stress in diabetic patients. *Diabetes Res. Clin. Pract.* **50**: S1.

Trainor, B.C. & Hofmann, H.A. 2006. Somatostatin regulates aggressive behavior in an African cichlid fish. *Endocrinology* **147**: 5119–5125.

Van Doorn, G.S., Edelaar, P. & Weissing, F.J. 2009. On the origin of species by natural and sexual selection. *Science*, **326**: 1704–1707.

Vandermeer, J., Evans, M.A., Foster, P., Hook, T., Reiskind, M. & Wund, M. 2002. Increased competition may promote species coexistence. *Proc. Natl Acad. Sci. USA* **99**: 8731–8736.

Von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. Lond. B* **266**: 1–12.

Wendelaar Bonga, S.E. 1997. The stress response in fish. *Physiol. Rev.* **77**: 591–625.

West-Eberhard, M.J. 1979. Sexual selection, social competition, and evolution. *Proc. Amer. Phil. Soc.* **51**: 222–234.

Wingfield, J.C., Hegner, R.E., Dufty, A.M. & Ball, G.F. 1990. The 'challenge hypothesis': theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *Am. Nat.* **136**: 829–846.

Witte–Maas, E. & Witte, F. 1985. *Haplochromis nyererei, A New Cichlid Fish from Lake Victoria Named in Honour of Mwalimu Julius Nyerere, President of Tanzania*. Brill, Leiden.

Zera, A.J. & Harshman, L.G. 2001. Physiology of life history trade-offs in animals *Ann. Rev. Ecol. Evol. Syst.* **32**: 95–106.

Zera, A.J., Harshman, L.G. & Williams, T.D. 2007. Evolutionary endocrinology: the developing synthesis between endocrinology and evolutionary genetics. *Ann. Rev. Ecol. Evol. Syst.* **38**: 793–817.

Received 25 April 2011; revised 12 August 2011; accepted 15 August 2011